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Award Number: W81XWH-04-1-0739

TITLE: Use of Nascent RNA Microarrays to Study Inducible Gene Expression in Breast Cancer Cells

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REPORT DATE: September 2005

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-09-2005	Final	15 Aug 2004 – 14 Aug 2005

4. TITLE AND SUBTITLE
Use of Nascent RNA Microarrays to Study Inducible Gene Expression in Breast Cancer Cells

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0739

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Mats Ljungman

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Michigan
Ann Arbor, MI 48109

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

To specifically analyze inducible gene expression profiles, we have developed a novel approach based on the isolation of nascent RNA. Following the inducible treatment, nascent RNA is pulse-labeled with BrU and isolated from total RNA by use of BrdU-conjugated magnetic beads. The isolated nascent RNA is then prepared for expression profile analysis using Affymetrix chips or any other microarray platform. Using a cell line with a temperature-inducible p53 protein, the nascent RNA microarray technique coupled to Affymetrix gene chip analysis was able to within 3 hours detect a 28- and 26-fold induction of p21 and mdm2 mRNAs, respectively, verifying that this technique is sensitive and able to detect known p53-inducible genes. In addition, a number of genes not previously known to be p53-inducible were detected. We are currently studying the gene expression profile following ionizing radiation using the nascent RNA microarray technique. The novel nascent RNA microarray approach developed here using the funding provided by DOD have many advantages over the currently used approach using steady-state RNA to specifically measure inducible gene expression profiles.

15. SUBJECT TERMS
Inducible gene expression, technique development, microarray analysis

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	10	19b. TELEPHONE NUMBER *(include area code)*

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INTRODUCTION

DNA microarray technology has revolutionized the way we study gene expression in different biological systems. With this technique, gene expression profiles from two or more samples can be conveniently analyzed using isolated steady state RNA. While this approach works well when comparing the steady-state levels of gene expression of different samples, measurements of inducible gene expression responses may be compromised by the inclusion of pre-existing steady state RNA in the analysis.

In response to DNA-damaging agents, cells induce signal transduction pathways that ultimately lead to the reprogramming of the expression of certain genes. Previous studies of inducible gene expression profiles have used steady-state levels of RNA as starting material for DNA microarray experiments. Using steady-state levels of RNA for DNA microarray studies of inducible gene responses has the following potential shortcomings:

1) Most of the RNA isolated from cells has been synthesized prior to the exposure to the inducing agent and thus "dilutes" the analysis of inducible RNA synthesis. In order to observe measurable changes in gene expression, RNA cannot be isolated from cells until the "steady-state" levels of a particular RNA species have dropped or increased. This will work well for genes that are not expressed prior to treatment but are induced following treatment. However, detection of mRNA expression changes of a gene that is repressed by the treatment will only occur if the half-life of the mRNA is short enough to deplete its steady-state level enough to be measurable.
2) The necessity of having to allow for long enough time for the steady-state levels of RNA to change sufficiently may lead to cell cycle distribution changes induced by the treatment. Since gene expression profiles differ in different stages of the cells cycle, any observable change in gene expression caused by the inducing agent may be related to cell cycle distribution changes rather than to a true inducible response.

Many DNA-damaging agents induce lesions in the DNA template that can interfere with the elongation of transcription. As a consequence, total steady-state RNA levels will decrease with time of treatment. When RNA samples are prepared for DNA microarray analysis, equal amounts of total RNA rather than RNA from equal number of cells are typically used. This approach will introduce a bias in the analysis where small genes (less likely to contain a transcription-blocking lesion) will appear to be induced although they may not be. Thus, the DNA microarray analysis will pick out small genes as inducible, medium sized genes as unchanged and large genes as repressed.

In an attempt to overcome some of the shortcomings of using steady-state RNA for the analysis of inducible gene expression profiles, we have developed a new approach that specifically utilizes nascent RNA for analysis. Following the inducible treatment, nascent RNA is pulse-labeled with BrU and isolated from total RNA by use of BrdU-conjugated magnetic beads. The isolated nascent RNA is then prepared for expression profile analysis using Affymetrix chips or any other microarray platform.

We envision that this new approach will be useful for the analysis of gene expression profiles in cells under situations when the gene expression is either induced or repressed by for example by stress or growth stimuli. By using nascent RNA as starting material, we believe that the gene expression profile analysis will be more sensitive and accurate than when using steady state RNAs. If we are successful, this nascent RNA isolation approach may be the technique of choice when studying genome wide inducible gene expression profiles.

BODY

Task 1. Develop technique to isolate and label nascent mRNA, Months 1-3:

a. *Different types of beads (e.g. sepharose or magnetic) will be evaluated for background binding of radioactively labeled RNA isolated from cells. Different washing conditions will also be evaluated in an effort to find conditions at which background RNA binding could be held at a minimum.*
b. *Nascent RNA will be labeled by incubation of cells with BrU and captured using anti-BU antibodies. Different labeling times and BrU concentrations will be examined for maximum yield. RNA labeled* in vivo *with both [^{3}H]uridine and BrU will be used to assess how well the beads will bring down the radioactively labeled RNA. Furthermore, multiple rounds of incubation of the RNA sample with the anti-BU antibody-conjugated beads will inform us whether the amount of beads added are sufficient or limiting.*
c. *After this optimization of the technique we will measure the yield of isolated nascent RNA obtained from five 60 mm cell culture plates. In pilot experiments we can obtain about 0.5 µg of nascent RNA but after optimization of the capturing techniques we may be able to*

obtain more. We will evaluate the amplification efficiency in a few RNA amplification kits according to the instructions from the manufacturers.

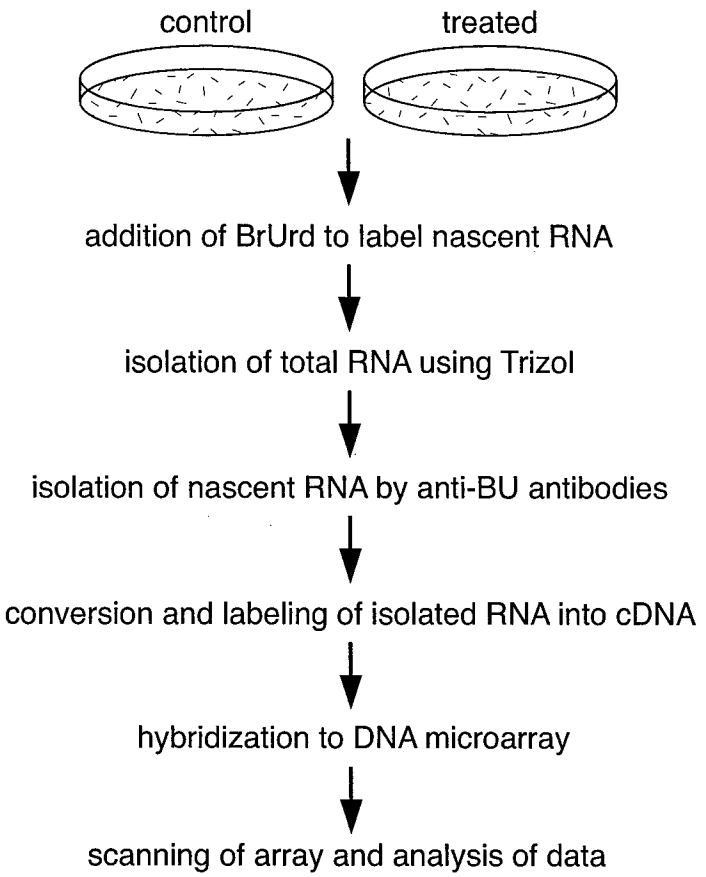

Figure 1. *Outline of the experimental procedure of the nascent RNA approach.*

We found that Dynabeads M-450 Goat anti-Mouse IgG from Dynal Biotech, Lake Success, NY gave the lowest background binding to labeled RNA. These beads were then used for the rest of the experiments. The background binding and specificity of nascent RNA isolation by the magnetic beads was evaluated by using RNA labeled with [^{3}H]uridine alone or RNA labeled with both [^{3}H]uridine and BrU. The RNA labeled with [^{3}H]uridine alone was used to measure non-specific binding to the beads and the double-labeled RNA was used to evaluate the specific binding. We found that less than 1% of the labeled RNA bound to the beads in a non-specific fashion. After optimizing the amount of beads and anti-BU antibodies used in the isolation reaction we were able to isolate more than 30% of the nascent RNA as determined by the fraction of radioactivity that was recovered by the antibody-conjugated beads in the experiments using RNA labeled with both BU and [^{3}H]uridine.

d. *The amplified RNA will be divided in two samples where one part will be labeled with Cy3 and the other part with Cy5.*
e. *The labeled RNA samples are then mixed and hybridized to a test cDNA array and analyzed according to instructions from the Microarray Core at the Comprehensive Cancer Center of the University of Michigan.*

We have changed from cDNA arrays to the Affymetrix microarray platform since the cost of this technology has gone down and because the microarray core at University of Michigan has a lot of experience using the Affymetrix platform.

Task 2. Compare the technique using nascent RNA with technique using steady state RNA, Months 4-6:

a. *Breast cancer cells will be mock-irradiated or irradiated with 5 Gy of ionizing radiation and nascent RNA labeled with BrU for 1-2 hours.*
b. *Total RNA will be isolated from the two groups and 1/10 of these samples are put aside and labeled "steady state RNA". The other half will be used to isolate nascent RNA.*
c. *Following amplification and labeling of the samples obtained from mock-irradiated cells with Cy3 and the samples from the irradiated cells with Cy5, the gene expression profiles will be analyzed using the human 4.1K cancer array available from our microarray core facility.*
d. *The radiation-induced gene expression profiles using the nascent RNA approach will be compared with the profile obtained using steady state RNA.*
e. *When we feel that the technique is working satisfactory using the cDNA microarrays, we will subject our nascent RNA preparations for analysis using the Affymetrix platform of microarrays (available at a reduced cost at the Microarray Core at the University of Michigan Comprehensive Cancer Center).*

To verify that the nascent RNA approach can be used to detect inducible gene expression following activation of a transcription factor we used the p53 mutant lung cancer cell line H1299/tsp53 expressing a temperature-sensitive p53 gene and a control cell line H1299/neo expressing a neo control vector. To activate the transcription factor p53 we lowered the temperature from 38°C (non-permissive temperature) to 32°C (permissive temperature). BrU was added to the cell cultures one hour after the switch and the cells were harvested 3 hours after the temperature switch. Following isolation of total RNA using the Trizol protocol, nascent RNA was isolated by incubation with the anti-BU-conjugated magnetic beads for 1 hour with agitation at room temperature. The isolated RNA (~1 µg) was subjected to one round of linear amplification and cDNA synthesis at which the samples were labeled with biotin for the Affymetrix array hybridization. The

Table I. Top-ten list of p53-inducible genes

Gene	Fold induction*
Alstrom syndrome 1	37
Hypothetical protein FLJ12377	31
Plexin C1	30
LYRIC/3D3	28
p21	28
Mdm2	26
Stem cell growth factor	21
cAMP responsive element modulator	18
MADS box transcription enhancer 2	17
jagged 1 (Alagille syndrome)	16

*Nascent RNA gene expression in H1299+tsp53 cells / nascent RNA gene expression in H1299+neo cells. Nascent RNA was collected 3 hours after switching to the permissive temperature of 32°C.

linear amplification, labeling, hybridization and reading of the results were performed by the microarray core facility at the University of Michigan Comprehensive Cancer Center.

Using the nascent RNA approach coupled to a 22k Affymetrix array (Fig 1) we found that 893 genes were up-regulated more than twofold and 818 were down-regulated more than twofold in a p53-dependent manner within 3-hours of shifting to the permissive temperature of 32°C. Two known p53-inducble genes, p21 and mdm2, were both recorded to be up-regulated more than 25-fold verifying that the nascent RNA approach is accurate and sensitive (Table I). We also found a number of genes that were dramatically down-regulated in a p53-

Table II. The top-ten most repressed genes by p53

Gene	Fold repression*
tachykinin, precursor 1	52
glut3	41
membrane metallo-endopeptidase	39
galactosylceramidase	23
cadherin 2	23
interlukin 13 receptor	22
microtubule-associated protein 1B	21
p57 (Kip2)	21
DEAD-box polypeptide 6	18
CGI-146 protein	16

*Nascent RNA gene expression in H1299+neo cells / nascent RNA gene expression in H1299+tsp53 cells. Nascent RNA was collected 3 hours after switching to the permissive temperature of 32°C.

dependent manner (Table II). Many of these genes have not previously been reported to be p53-regulated.
We confirmed the p53-dependent induction of p21 found with our nascent RNA approach using RT-PCR of the isolated nascent RNA (Fig 2). Using GAPDH as an internal control we found that p21 expression went up about 240% in the in the H1299/tsp53 cells compared to the H1299/neo cells when using the nascent RNA preparation. When performing RT-PCR using a sample of total steady state RNA from the same experiment we found a 180% induction of p21. Although our PCR procedure do not give us quantitative results, it supports the microarray data that the nascent RNA approach is detecting the expected p53-mediated p21 induction and that the sensitivity of the nascent RNA approach may be higher than the traditional steady-state RNA approach.

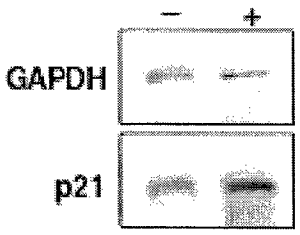

Figure 2. *H1299/neo (p53 -) and H1299/tsp53 (p53 +) were switched to the permissive temperature and nascent RNA was isolated 3 hours later as described in the text. The nascent RNA was subjected to reverse transcription and 20 cycles of PCR using specific primers for GAPDH and p21. Phosphoimage shows that p21 is up-regulated about 240% in the H1299/tsp53 cells compared to the neo*

Task 3. Data collection from radiation sensitive and resistant breast cancer cells, Months 7-12:

a. *The radiation sensitivity of a panel of breast cancer cell lines obtained from Dr. Steve Ethier, who is the director of the breast cancer cell line repository at the University of Michigan Comprehensive Cancer Center, will be assessed using both apoptotic and clonogenic survival assays.*
b. *Radiation-induced gene expression profiles of these cells will be obtained using the nascent RNA technique.*
c. *The radiation-inducible gene expression profiles will by analyzed and any expression pattern associating with sensitivity or resistance will be noted.*
d. *The long term goal will be to assess whether the radiation-induced gene expression profile alone could predict whether a cell line (or a tumor) would be radiation sensitive or resistant. This could be applied to any treatment and may ultimately aid in tailoring the treatment for individual tumors.*

The studies presented in Task #3 are ongoing and we do not have any data collected yet. Before evaluating the radiation-inducible expression of the different breast cancer cell lines described in Task #3, we decided to investigating the radiation-induced expression profiles in two isogenic cell lines differing only in

their p53 status. These cell lines are HCT116 (wtp53) and HCT116 (p53 null) and was provided to us by Dr. Bert Vogelstein, Johns Hopkins Medical School. The reason for this is that many of the changes observed in gene expression occurring following exposure to ionizing radiation would be expected to be mediated by p53. By investigating the gene expression profiles both in p53 wild-type and p53 null cells we will be able to discriminate between radiation-induced gene expression mediated by p53 and p53-independent radiation-induced gene expression. These studies are under way.

KEY RESEARCH ACCOMPLISHMENTS

- We have successfully developed a new technique to study inducible gene expression using nascent RNA instead of steady-state RNA.

- This technique was applied to a 22k Affymetrix microarray platform and the results from these studies using the nascent RNA fraction show that this technique was able to detect the expected induction of p21 and Mdm2 following induction of the transcription factor p53.

- RT-PCR of the p21 gene confirmed the results found on the array and suggested that the nascent RNA approach is more sensitive than the steady state approach even for a rapidly up-regulated gene such as p21.

- The nascent RNA approach revealed the p53-mediated induction/repression of a number of genes not previously known to be p53-regulated. These findings need to be verified in follow-up studies.

REPORTABLE OUTCOMES

We have developed a novel technique to study inducible gene expression profiling using nascent RNA instead of steady state RNA. We are in the process of exploring whether we can seek a patent for this new technique. We are also in the process of writing up our findings in a manuscript. This work was presented at the Era of Hope Conference in Philadelphia in June 2005. We are planning to seek NHI/NCI funding for studies in the near future using this new technique.

CONCLUSIONS

There is great interest in the many uses of DNA microarray technology in medicine, epidemiology, bioterrorism and in the environmental sciences just to name a few disciplines. The DNA microarray technique measures how much a particular gene is used by the cells in any tissue of interest. Since the activity of thousands of genes can be measured simultaneously, specific "expression profiles" can be generated that can help us determine the origin of the tissue and whether the tissue is healthy or diseased. This technique has also been used to

study the effect certain environmental exposures or treatments with anti-cancer agents have on the expression profile of cells. By studying what genes the cells use more or less following a certain type of treatment we can gain important information about what cellular responses to that type of exposure. Furthermore, we may be able to learn why certain tumor cells do not respond to a certain type of anti-cancer agents.

In this study we developed a new approach that we think will improve the accuracy and sensitivity of the microarray technology when studying inducible gene expression responses. The basis of this novel approach is to specifically analyze changes occurring by the treatment itself without taking into account how much expression of the different genes was occurring prior to the treatment. We are currently using this approach to study radiation-induced gene expression in breast cancer cell lines and initiate the identification of predictable markers for sensitivity to radiation or chemotherapy. These studies may lead to clinical application for the evaluation of responses to radiation or chemotherapy in tumors and/or in normal tissues/cells.